REMARKS

Applicants have amended claim 28 to correct an inadvertent clerical error.

Claims 1-4 and 28-31 stand rejected under 35 U.S.C. §112, first paragraph for purportedly failing to comply with the enablement requirement. Applicants respectfully disagree.

> "A decision on the issue of enablement require[s] determination of whether a person skilled in the pertinent art, using the knowledge available to such a person and the disclosure in the patent document, could make and use the invention without undue experimentation. It is not fatal if some experimentation is needed, for the patent document is not intended to be a production specification."
>
> Northern Telecom, In. v. Datapoint Corp., 15 USPQ 1321, 1229

Claim 1 describes an isolated peptide consisting of a particular amino acid sequence, i.e., ALKDVEERX (SEQ ID NO: 1) wherein X may be any amino acid. Independent claim 2 describes the claimed peptides with even more particularity in that the amino acid "X" in ALKDVEERX (SEQ ID NO: 1) is defined as a particular subset of amino acids, i.e., Ala, Val, Leu, Ile, Pro, Phe, Met, Trp or Glu. Claim 3, which depends on claim 1, specifies the sequence of the peptide as "consisting of SEQ ID NO: 3 or SEQ ID NO: 5." It would require only routine experimentation by one of ordinary skill in the art to make any peptide described by these claims. Claim 4 describes a composition useful in provoking a cytolytic T cell response comprising the isolated peptide of claim 1 and an adjuvant. One of ordinary skill in the art in the relevant field would readily understand and easily make such a composition without undue experimentation.

The Examiner contends that the specification cannot be reasonably extrapolated to enable the claims because one of skill in the art could not predict that MAGE-C2 protein is expressed in primary tumor cells and thus one of skill in the art would not know how to use the invention (Office Action page 5).

Applicants respectfully disagree and direct the Examiner's attention to the references of Li et al, Lab.Invest. 83:1185-1192 (2003) and Zhuang et al., Cancer Immunity 6:7 (5 April 2006) (enclosed) who demonstrate that MAGE-C2 protein (also known as CT10, HCA587, MAGE-C2, MAGEE1 and MGC13377) is expressed in normal adult testes, cancer cell lines and frozen primary tumor samples and as such MAGE-C2 is expressed in primary tumors. Furthermore, one of skill in the art would thus know how to use the claimed peptides regardless of whether all primary tumor cells express MAGE-C2.

Applicants have taught that the claimed peptides are useful for determining if a cell expresses an HLA molecule, particularly an HLA-A2 molecule (See e.g., page 11, lines 19 to page 12, line 2) and that the peptides are useful for determining if a sample comprises a CTL specific for complexes of HLA-A2 and the peptide (see page 12, line 26 to page 13, line 3). Li et al. *supra*, Zhuang et al. *supra* demonstrate that MAGE-C2 is expressed in tumors. Thus, one of skill in the art presented with Applicants' specification would readily make the claimed peptides and compositions, and use them to determine if a cell expresses HLA-A2.

Regarding the rejection of claims 28-31 for purported lack of enablement, claims 28-30 relate to an isolated complex useful in isolating cytolytic T cells, wherein the isolated complex comprises a first and second binding partner that are specific for each other, the second binding partner is bound to a plurality of tetramers of an HLA-A2 molecule, a β_2 microglobulin and the peptide of claim 1. Claim 31 relates to a composition comprising the complex of claim 28 and an adjuvant. As discussed *supra*, one of ordinary skill in the art can readily prepare the peptides of claim 1. Those of ordinary skill in the relevant art are also well versed in the preparation of the tetramers and in addition, applicants have described how such tetramers are prepared, see e.g., page 13, line 29 to page 14, line 7. Adjuvants are also well known to, and routinely used by, those of skill in the art and Applicants have also disclosed examples of adjuvants that are useful in this invention (see specification page, 14, lines 24-25). As such, Applicants have taught those of skill in the art <u>how to make</u> the isolated complexes of this invention.

Applicants have also taught those of skill in the art <u>how to use</u> the complexes of this invention; See e.g., specification page 14, line 27 to page 15, line 2, where Applicants have taught that the tetramers may be used to identify or stimulate CTLs, which recognize specific

HLA/peptide complexes, in peripheral blood samples, by admixing the sample with the complex described above, and identifying or isolating CTLs that bind thereto. Applicants have also taught that the complexes may be used in methods for determining the presence of a CTL in a sample, e.g., one may assay for proliferation of the CTL cells responding to the complex, or one may assay a sample admixed with the complex for lysis of cells expressing a complex of the peptide and an HLA molecule; One may assay for the release of TNF by the CTLs. Applicants have taught "MAGE-C2 is expressed in about 40% of melanomas and also other types of tumors (Lucas, Int. J. Cancer: 87, 55-60, 2002)" (specification page 48, lines 18-19; page 40, line 27 to page 41, line 1) and although the Examiner contends that expression of MAGE-C2 RNA is not sufficient to establish that a MAGE-C2 polypeptide is expressed in primary tumor cells, one of skill in the art at the time of filing would have reasonably expected that for such a member of the MAGE family of genes a polypeptide would be expressed in normal testes and primary tumor cells. In fact this expectation was confirmed by Li et el., *supra* and Zhuang et al. *supra* who demonstrated that MAGE-C2 protein (also known as CT10, HCA587, MAGE-C2, MAGEE1 and MGC13377) is expressed in normal adult testes, cancer cell lines and frozen primary tumor samples. As such those of skill in the relevant art would expect that the peptides and complexes described in the present application would be used successfully as disclosed by Applicants.

The foregoing remarks demonstrate that Applicants have provided sufficient teaching for one of skill in the art to make and/or use the peptides and compositions of this invention and thus have satisfied the requirements of 35 U.S.C. 112, first paragraph. As such, Applicants respectfully request that the Examiner reconsider and withdraw her rejection of the claims.

The Examiner also contends that claims 1-4 and 28-31 are not enabled because, in the Examiner's opinion, the specification, while being enabling for an isolated peptide consisting of the amino acid sequence of ALKDVEERV (SEQ ID NO: 3) or a complex comprising an isolated peptide consisting of the amino acid sequence of SEQ ID NO: 3, is not enabling for an isolated peptide consisting of an amino acid sequence of ALKDVEERX (SEQ ID NO: 1) wherein X is Ala, Leu, Ile, Pro, Phe, Met, Trp or Glu or a complex comprising such peptide. In particular the Examiner contends the specification cannot be reasonably extrapolated to enable the scope of the claims because one of skill in the art could not predictably use the peptide of ALKDVEERX

(SEQ ID NO: 1). Applicants respectfully disagree. Enablement does not require the claims to exclude possible inoperative substances (*In re Dinh-Nguyen* 492 F.2d 856, 858-59, 181 USPQ 46, 48 (CCPA 1973)) and does not preclude some experimentation,

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>
> Northern Telecom, In. v. Datapoint Corp., 15 USPQ 1321, 1229

With Applicants' specification in hand, a person skilled in the pertinent art, using the knowledge available to such person and the disclosure of Applicants' specification, could make and use the peptides of claims 1-4 and the tetramers of claims 28-31 without undue experimentation. Applicants have provided sufficient guidance for one of skill in the art to ***make*** such peptides and tetramers (see e.g., page 13, lines 29-31) and those of skill in the art at the time this application was filed could readily make the peptides and tetramers using routine methods available in the art. Regarding the use of the claimed peptides, Applicants have provided sufficient guidance for one of skill in the art to ***use*** the peptides and tetramers (see e.g., specification page 12, line 26 to page 13, line 3 and page 14, line 29 to page 15, line 2, as discussed supra) and have disclosed how to predict the ligation strength of the peptides for an HLA molecule by using the SYFPEITHI database at syfpeithi.bmi-heidelberg.com and the BIMAS database at bimas.dcrt.nih.gov/molbio/hlabind/index.html (Parker, K. C., et al., *J. Immunol.* 152:163., 1994) (specification page 46, lines 8-11).

The Examiner contends that one of skill in the art could not predictably use the peptide ALKDVEERX (SEQ ID NO: 1) wherein X is any amino acid other than valine because the claims encompass peptides wherein X may be amino acids having widely divergent shapes and sizes and that differ in charge status. The Examiner contends:

> "George et al. (2005 Trends in Immunology 26(12):653-659) teach that the specificity of the interaction with which a T-cell receptor

> recognizes antigen in the form of a peptide held in the groove of an MHC class molecule is such that a single amino acid substitutions can abolish the ability of T cells to respond to the antigen or can convert the peptide to an antagonist peptide that "turns off" the ability of a population of T cells to respond by proliferation."
>
> (Office Action page 9).

However, George et al. is discussing a different peptide than those claimed herein, and George fails to disclose which amino acids in that other peptide were substituted. George also fails to disclose the frequency with which any substitution affected the ability of a population of T cells to respond by proliferation. In fact, George et al. is discussing a peptide disclosed in another journal article, Sloane-Lancaster et al, *Nature* 363:156-159 (1993)("Sloane-Lancaster") (enclosed). Sloane-Lancaster mutated the center amino acid (position 70) in the hemoglobin β^d minor chain peptide Hbβ(64-76), which Sloane-Lancaster states was known to be the contact amino acid of that peptide, (see page 156, left col., 2d paragraph, 2d sentence). In contrast, Applicants' claimed peptides only vary at the terminal 9^{th} position, and those of skill in the art appreciate, and Applicants have disclosed, that for the NY-ESO-1 tumor antigen, a T-cell clone retained its recognition after substitution of the terminal position 9 amino acid (specification page 26, lines 10-12). Furthermore, one of skill in the art can reasonably predict based on Applicants' disclosure, the binding of the claimed peptides to HLA and easily assay their recognition by CTLs. Thus one of skill in the art based on Applicants' disclosure could predictably use of particular peptides and complexes as claimed without resorting to undue experimentation.

In view of the foregoing remarks, Applicants respectfully request that the Examiner reconsider and withdraw the rejections of the claims under 35 USC 112, first paragraph.

Applicants believe no additional fee is due with this response. However, if an additional fee is due, please charge our Deposit Account No. 06-2375, under Order No. WO-LUD 5780.2/10312064 from which the undersigned is authorized to draw.

Dated: Aug. 24, 2006

Respectfully submitted,

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Cancer Immunity, Vol. 6, p. 7 (5 April 2006)
Submitted: 19 January 2006. Accepted: 27 January 2006.
Contributed by: LJ Old

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CANCER IMMUNITY
Article

Generation of monoclonal antibodies to cancer/testis (CT) antigen CT10/MAGE-C2

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Keywords: cancer/testis, tumor antigens, CT10/MAGE-C2, monoclonal antibodies, ELISA, immunohistochemistry

CT10/MAGE-C2 is a recently identified antigen that, typically of cancer/testis (CT) antigens, can be found in various malignant tumors and in normal adult testis. As with many other CT antigens, our knowledge is based mainly on mRNA expression data. In the present study, we describe the generation of mAbs to CT10/MAGE-C2 for the analysis of its protein expression. Newly generated clones were chosen based on their reactivity in ELISA, immunoblotting, and immunohistochemistry (IHC). Emphasis was put on the reactivity of newly generated reagents on formalin-fixed, paraffin-embedded tissue to ensure their applicability to archival material. Eventually we selected two clones, LX-CT10.5 and LX-CT10.9, that showed intense reactivity to CT10/MAGE-C2 protein and *CT10/MAGE-C2* mRNA-positive cell lines, but no cross-reactivity with other CT antigens. Both mAbs show superior staining characteristics in IHC and are applicable to frozen and paraffin sections. In testis, CT10/MAGE-C2 displays the typical CT pattern with regard to staining of germ cells, which is intense during the early maturation stages. In tumors, we analyzed a limited number of cases displaying the typical heterogeneous CT expression pattern. Interestingly, immunoreactivity was seen solely in the nucleus: No staining was seen in the cytoplasm of tumor cells.

Introduction

CT antigens are found in various types of malignant tumors, whereas in normal adult tissues, their expression is generally restricted to testicular germ cells. To date, over 40 CT genes or gene families have been identified, with *MAGE-A1* being the prototype (1, 2, 3). CT antigens can elicit autologous immune responses in tumor patients, and immunological methods such as autologous T cell assays and SEREX (serological analysis of recombinant cDNA expression libraries) have been used to identify many CT antigens (4, 5, 6, 7). Other techniques that were used for CT gene identification include representational-difference analysis and database mining (8, 9).

CT10/MAGE-C2, a novel CT antigen, was recently identified in melanoma cell line SK-MEL-37 by representational-difference analysis, a PCR-based subtractive hybridization method that isolates differently expressed genes by comparing the expression profile of two cDNA libraries (8, 10). The *CT10/MAGE-C2* gene shows significant homology to *CT7/MAGE-C1* and exhibits a typical CT antigen mRNA expression pattern. *CT10/MAGE-C2* maps to chromosome Xq27, in close proximity to the *CT7/MAGE-C1* and *MAGE-A* genes (8). Moreover, *CT10/MAGE-C2* has a 3-bp difference to *MAGE-C2* and *HCA587*, which have identical cDNA sequences (11). *HCA587* mRNA has been detected in 59/105 (56%) of hepatocellular carcinoma patients tested (12). T lymphocyte precursors recognizing antigens encoded by *MAGE-C2* have been identified both before and after vaccination with MAGE-A antigens in the recent evaluation of several small-scale therapeutic MAGE-A protein or peptide vaccines. In one of the vaccinated patients, a majority of the antitumor CTL clones present recognized *MAGE-C2*-encoded antigens (13, 14). However, to date no data are available concerning CT10/MAGE-C2 expression at the protein level in either normal or tumor tissues. Consequently, in the present study we address this lack of knowledge by describing the generation of two mAbs to CT10/MAGE-C2 (LX-CT10.5 and LX-CT10.9) and their application for protein expression analysis in a limited number of tissues.

Results

After BALB/c mice were immunized with CT10/MAGE-C2 protein, spleen cell fusions were undertaken and hybridoma supernatants were screened by indirect solid phase ELISA (Figure 1). A total of 11 clones (LX-CT10.1 to LX-CT10.12) were positive for CT10/MAGE-C2 and negative for NY-ESO-1, SSX2, MAGE-A3, CT7, and CT16 (Figure 1).

Western blot analysis was used to assess antibodies for recognition of CT10/MAGE-C2 antigen that is naturally expressed in human tumor cell lines. The cell lines used in this analysis were pretyped for *CT10/MAGE-C2* RNA expression by RT-PCR. Monoclonal Abs LX-CT10.5 and LX-CT10.9 recognized a protein with an apparent molecular weight of 49 kDa in NP-40 detergent lysates of *CT10/MAGE-C2* expressing human melanoma cell lines (SK-MEL-37 and SK-MEL-55). This reactivity was not observed with a detergent lysate of a human melanoma cell line (SK-MEL-24) which does not express *CT10/MAGE-C2*. The 49 kDa protein co-migrated with the *E. coli*-derived recombinant CT10/MAGE-C2 protein recognized by those antibodies (Figure 2).

The positive clones were then tested for reactivity by IHC employing various antigen-retrieval techniques. Testis with

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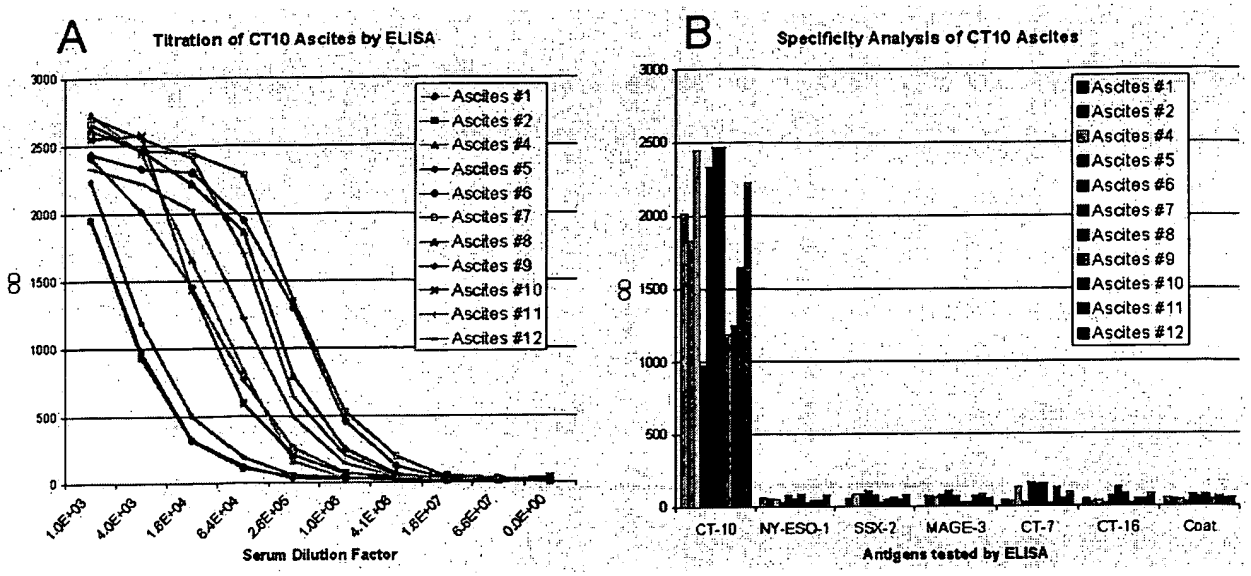
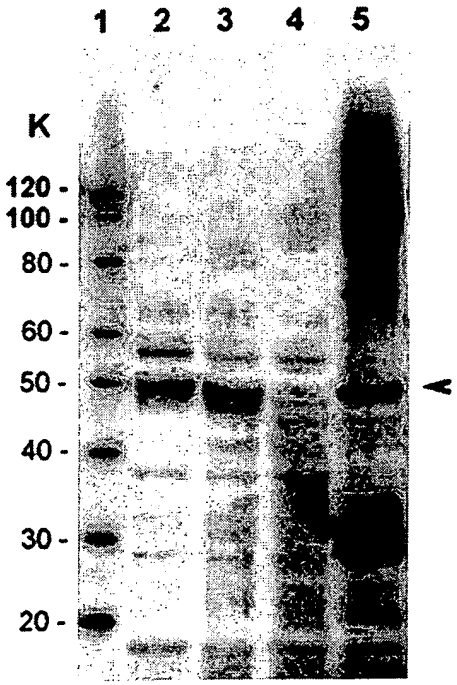
Figure 1



Solid phase ELISA of ascites fluid from 11 newly generated anti-CT10/MAGE-C2 clones (LX-CT10.1 to LX-CT10.12). (A) Titration assay using ascites from clones LX-CT10.1 to LX-CT10.12 and full-length recombinant CT10/MAGE-C2 protein. (B) Specificity analysis showing reactivity with CT10/MAGE-C2 and no reactivity with CT antigens NY-ESO-1, SSX2, MAGE-A3, CT7, and CT16.

Figure 2



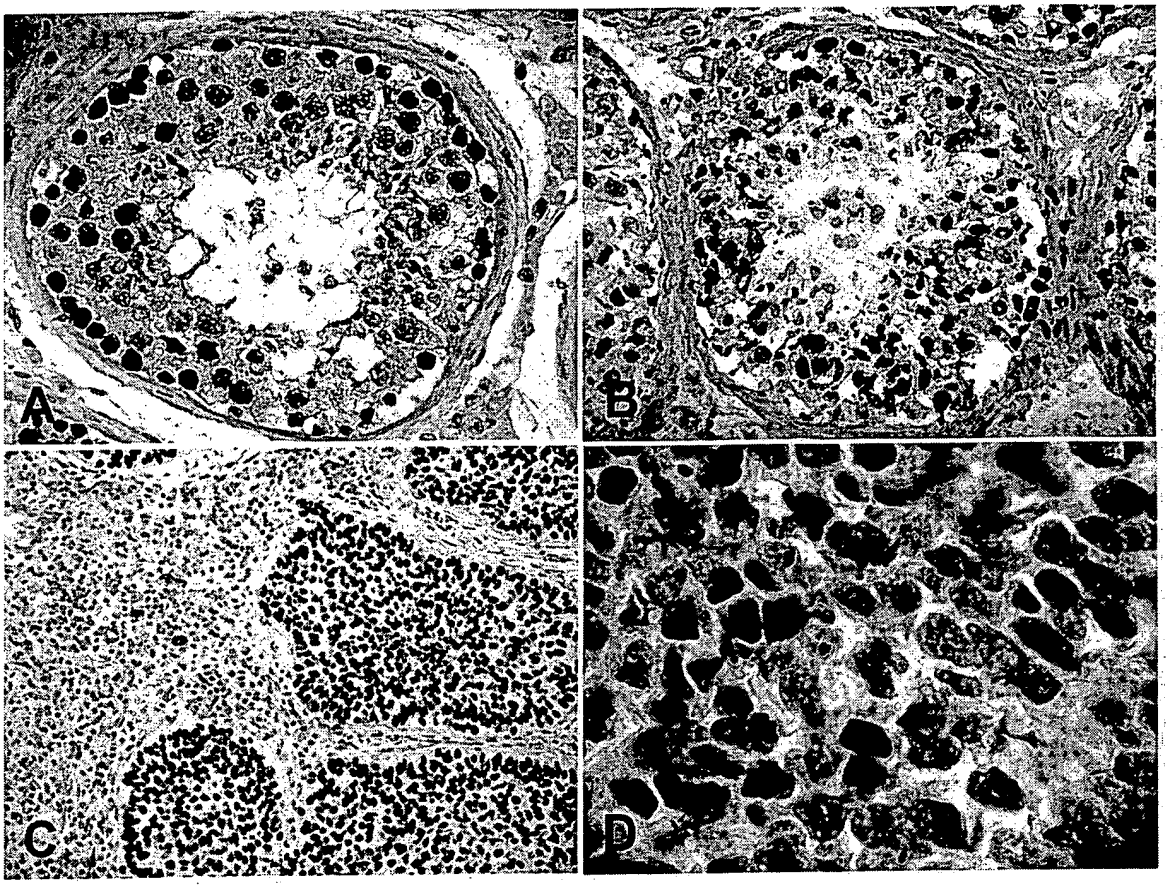
Immunoblotting of human cancer cell lines pretyped by RT-PCR for *CT10/MAGE-C2* mRNA expression with mAb LX-CT10.5 (NP-40 detergent lysates, chemiluminescence detection). Positive reaction of mAb LX-CT10.5 with a protein of an apparent molecular weight of 49 kDa (lane 1) in lysates of *CT10/MAGE-C2* mRNA-positive melanoma cell lines SK-MEL-37 (lane 2) and SK-MEL-55 (lane 3); no reactivity was seen with the *CT10/MAGE-C2* mRNA-negative melanoma cell line SK-MEL-24 (lane 4); the proteins detected by mAb LX-CT10.5 co-migrate with the 49 kDa *E. coli*-derived recombinant CT10/MAGE-C2 protein (arrow, lane 5).

preserved spermatogenesis served as an initial test tissue. Clones LX-CT10.4, LX-CT10.5, LX-CT10.9, and LX-CT10.10 showed superior staining of testicular germ cells in the typical CT antigen pattern (Figure 3). Subsequent IHC analysis of snap-frozen testicular tissue showed immunoreactivity of clones LX-CT10.5 and LX-CT10.9 only; LX-CT10.4 and LX-CT10.10 were negative. Consequently, clones LX-CT10.5 and LX-CT10.9 were used for further analysis (Figure 3). Both clones showed a similar staining pattern that was optimal using DAKO hipH antigen retrieval solution. In testicular tissues, both clones had intense nuclear staining of the spermatogonia and less intense staining of spermatocytes. No staining was seen in Sertoli cells or interstitial tissue. In an RT-PCR/IHC parallel analysis, 28 lung tumor specimens were studied side by side for mRNA and protein expression. Twenty tissues were *CT10/MAGE-C2* mRNA-positive, and eight tissues were *CT10/MAGE-C2* mRNA-negative. In 20 cases, RT-PCR and immunostaining were congruent (RT-PCR/IHC: 13x +/+, 7x -/-); 8 cases showed discrepant results (RT-PCR/IHC: 7x +/-; 1x -/+). In all analyzed tissues, immunostaining was exclusively nuclear (Figure 3). The staining was mostly heterogeneous, involving only portions of the tumor. No significant staining was seen in the cytoplasm or in any normal tissue adjacent to the tumor. In the tissue microarray of normal tissues, staining was seen in testicular tissue, but not in any other tissue (esophagus, stomach, duodenum, ileum, colon, liver, gall bladder, pancreas, mesentery, thyroid gland, salivary gland, adipose tissue, skeletal muscle, adrenal gland, lymph node, peripheral nerve, thymus, spleen, tonsil, lung, prostate, kidney, urinary bladder, mammary gland, skin).

Discussion

Although they were originally identified in melanoma, CT antigens have since been found in a wide array of tumors, but not in normal tissues except for germ cells and, occasionally, placenta (15). Due to this expression pattern, and due to their

Figure 3



IHC staining of normal adult testis (A, B) and lung carcinoma (C, D) using mAb LX-CT10.5 (biotinylated secondary Ab, ABC method, DAB chromogen). (A) Formalin-fixed, paraffin-embedded testis tissue with intense nuclear staining of intratubular germ cells and showing no staining of interstitial tissue and intratubular Sertoli cells. (B) Acetone-fixed frozen testis tissue with a similar staining pattern. (C) Lung carcinoma with homogeneous nuclear staining of tumor areas. (D) High-power magnification of lung carcinoma showing negative and intense nuclear staining of cancer cells.

ability to elicit either cytotoxic T cell or serological immune responses in the autologous host, CT antigens have generated considerable interest as targets for cancer immunotherapy (2, 16, 17). Several early-phase clinical trials employing CT antigens have been initiated or completed. Immune responses were clearly demonstrated and were indicative of clinical efficacy (18, 19, 20). For an accurate assessment of the suitability of individual CT antigens as potential vaccines, and subsequently as an eligibility criterion for patient entry into clinical trials, it is essential that the antigen distribution within, and between, tumors be determined using specific and carefully characterized mAbs. Due to the techniques currently employed to isolate CT antigens, information about their mRNA expression is readily available, but protein expression analyses require the laborious and time-consuming generation of serological reagents and are hence scarce. Consequently, much less is known about the actual expression of these antigens at the protein level. Monoclonal Abs have been developed for only some of the more than 40 CT antigens, such as MAGE-A1, MAGE-A4, MAGE-A11, CT7/MAGE-C1, and NY-ESO-1 (18, 19, 20, 21, 22, 23, 24, 25, 26). In the present study, we report the generation of a mAb to CT10/MAGE-C2, a recently identified CT antigen.

As a result of our prior experience in generating mAbs against CT antigens, the present study uses several approaches to demonstrate mAb specificity, including ELISA, Western blot assays, and IHC analysis of RT-PCR pretyped tumor tissues.

In normal tissues, we found CT10/MAGE-C2 protein expression to be consistent with that generally exhibited by CT antigens, with antibody reactivity restricted to testis among the normal adult tissues assayed. As with other CT antigens encoded on the X chromosome, such as NY-ESO-1 and the MAGE-A antigens, CT10/MAGE-C2 protein is most abundant in spermatogonia and is present at lower amounts in later-stage germ cells such as spermatocytes (21, 22, 27).

In the context of cancer, we analyzed a limited number of RT-PCR pretyped lung tumor specimens. The majority of specimens show concordance between protein and mRNA expression. However, in a number of samples, the RT-PCR data did not agree with those generated by IHC. Similar discrepancies between mRNA and protein expression had previously been found for several other CT antigens (21, 22, 27, 28). This appears to be largely due to the heterogeneous expression of CT antigens in tumor tissue. In contrast, an almost complete concordance of protein and mRNA expression is generally seen for melanocyte differentiation antigens such as tyrosinase and Melan-A (MART-1), probably due to the much more continuous nature of their expression in tumors (29, 30, 31). Also, it is possible that the newly generated antibodies may be identifying an

alternatively spliced protein or a truncated protein that the primers used in the present study were not able to detect. In this context, it should be kept in mind that the members of several CT antigen families show a high degree of homology, and cross-reactivities of the serological reagents cannot be fully excluded. In the limited number of tumor specimens we examined, CT10/MAGE-C2 protein was restricted to the nucleus of neoplastic cells. This contrasts with NY-ESO-1 and with most MAGE-A antigens, which are predominantly present in the cell cytoplasm. However, MAGE-A11 is also exclusively present in the nucleus in cancer cells (25).

Interestingly, at a molecular level CT10 shares significant similarity to CT7 which is typically expressed in the cytoplasm and nucleus of tumor cells (8, 24). Little is known about the biological function of CT antigens, although recent data indicate that CT antigens can act as co-repressors or co-activators of gene expression by interacting with DNA-binding proteins. In this context, the presence of CT10/MAGE-C2 in the nucleus is especially intriguing.

In the present study we report the generation of new mAbs to CT10/MAGE-C2. A preliminary IHC analysis confirms a typical CT expression pattern and suggests, on a cellular level, an exclusive nuclear localization of the protein. Further studies of the expression and localization of the CT10/MAGE-C2 protein are now possible and are necessary in order to analyze its presence in various tumors and help to clarify its biological function.

Abbreviations

CT, cancer/testis; IHC, immunohistochemistry

Acknowledgements

This work was supported by the James R. Kerr Program of the Ludwig Institute of Cancer Research and by the Chinese KBRSF (Grant No. 2001CB510004).

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Materials and methods

Antigen and ELISA

Full-length recombinant human (rh) CT10/MAGE-C2 protein (372 aa) was generated in *E. coli* as previously described (32). Briefly, the complete *CT10/MAGE-C2* coding region was cloned into pQE30 to generate an N-terminal His-tagged fusion protein.

For ELISA analysis of hybridoma supernatants, microtiter polystyrene plates (Nunc, Nagel Inc., Roskilde, Denmark) were coated with 5 μg/mL rhCT10/MAGE-C2 in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6), and incubated overnight at 4°C. Various dilutions of hybridoma supernatant were added. Detection of the primary antibody was done with a peroxidase-labeled goat-antimouse IgG Ab (1:2000; DAKO, Glostrup, Denmark). TMB substrate (Sigma, St. Louis, MO, USA) was used as chromogen. Results were analyzed at 450 nm on a microplate reader (BIO-RAD, Hercules, CA, USA).

Production of hybridomas

Purified rhCT10/MAGE-C2 protein was used to immunize BALB/c mice, and hybridomas were generated and cloned as previously described (32). In brief, mice were immunized three times with 20 μg rhCT10/MAGE-C2 protein at 3-wk intervals, followed by a booster injection 10 d after the final immunization. Complete Freund's adjuvant was used for the first injection; IFA was used thereafter, except for the booster injection, which was administered without adjuvant. Spleen cells from immunized mice were fused with SP2/0 mouse myeloma cells. Hybridomas were cultured in RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) and screened against the immunizing protein by solid phase ELISA. Positive hybridomas were subcloned four times by limiting dilution, and then screened again by ELISA. Positive clones were expanded in 24-well and 75-cm^2 flasks (Nunc, Nagel Inc., Roskilde, Denmark), or as ascites fluid after i.p. injection of hybridoma cells. The Ig isotype was identified using an isotype kit (Sigma, St. Louis, MO, USA, ISO-2).

Antibody purification

Monoclonal Abs were purified from supernatant or mouse ascites by protein G affinity chromatography as described by the manufacturer (Pierce, Rockford, IL, USA)

Western blot analysis

Human cancer cell lines were lysed with 0.5% NP-40 in 0.15 M NaCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.5 containing Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA). Samples of lysates containing 25 μg total protein and recombinant CT10/MAGE-C2 antigen (0.05 μg) were resolved on 4-12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) under reducing and nonreducing conditions. Proteins were blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA) and incubated overnight with 1 μg/ml primary antibody, followed by incubation with HRP-labeled goat-antimouse polyclonal antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Specific binding was visualized by chemiluminescence (Western Lightning Plus, Perkin Elmer Life Sciences, Boston, MA, USA).

IHC staining

Clones with anti-CT10/MAGE-C2 reactivity in ELISA and Western blot assays were then analyzed by IHC. The analysis was initiated using formalin-fixed, paraffin-embedded (FFPE) tissues to ensure applicability to standard archival material. Testis known to be *CT10/MAGE-C2* mRNA-positive was used as the initial test tissue. Each supernatant was analyzed at different dilutions using several heat-based antigen-retrieval methods. Five micron cuts of testis were applied to slides for IHC, deparaffinized, rehydrated in a series of graded alcohols, and then incubated in 3% H_2O_2 for 20 min to block endogenous peroxidase reactivity. Antigen retrieval was done using a vegetable steamer and various buffer solutions (citrate buffer, 10 mM, pH 6.0; EDTA buffer, 1 mM, pH 8.0; DAKO hipH buffer). The tissue sections were then incubated with the primary antibody in a humid chamber at 4°C overnight. Detection of the primary antibody was performed with a biotinylated horse-antimouse antibody (Vector, Burlingame, CA, USA) followed by an avidin-biotin-complex system (ABC-Vector, Elite, Vector, Burlingame, CA, USA). Diaminobenzidine tetrahydrochloride (DAB, BioGenex, San Ramon, CA, USA) was used as chromogen. The clones with superior staining were then titered again on testis tissue to achieve optimal results. Clones that were immunopositive on FFPE slides were then tested on frozen testis tissues. Additionally, these clones were tested on a limited number of tumor tissues pretyped by RT-PCR for *CT10/MAGE-C2* expression. Finally, a tissue microarray of normal tissues was tested for CT10/MAGE-C2 staining. All tissues were obtained in compliance with the legal regulations of the Department of Pathology of the Xijing Hospital, Xi'an, China and the Ludwig Institute of Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, USA.

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0023-6837/03/8308-1185$03.00/0
LABORATORY INVESTIGATION
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Vol. 83, No. 8, p. 1185, 2003
Printed in U.S.A.

HCA587 Antigen Expression in Normal Tissues and Cancers: Correlation with Tumor Differentiation in Hepatocellular Carcinoma

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SUMMARY: The *HCA587* gene, identified by serological analysis of recombinant cDNA expression library (SEREX) from a hepatocellular carcinoma (HCC) patient, encodes a new member of cancer-testis antigens. HCA587 mRNA expression in normal tissues and cancers has been previously reported. To estimate its immunogenicity to induce immune response, it is essential to analyze HCA587 expression at the protein level. In this study anti-HCA587 polyclonal antibody, termed "TC-1," was generated, and the expression of HCA587 protein was assessed by immunohistochemical staining in a panel of normal and tumor tissue sections. No HCA587 protein was shown in normal tissues except germ cells in testis and Purkinji cells in cerebellum. In HCC specimens the HCA587 protein was expressed in 37.1% (26 of 70) samples. The expressed protein was either located in the cytoplasm or nucleus depending on the individual samples. More importantly, there appears to be correlation between the tumor differentiation of HCC and HCA587 protein expression, ie, the lower differentiation, the higher percentage of protein expression. Coincidentally, seroreactivity showed that the Ab specific to recombinant HCA587 protein was detected only in the sera of three patients with poorly differentiated HCCs. HCA587 antigen was also expressed in different proportions in melanoma, lymphoma, pancreatic cancer, and lung cancer. (*Lab Invest 2003, 83:1185–1192*).

The recent advances of molecular technology for analyzing cellular and humoral immune response to cancer cells has led to the identification and characterization of a large number of human tumor-associated antigens recognized by $CD8^+$ T cells and antibodies (Harashima et al, 2001; Ito et al, 2001; Shichijo et al, 1998). Most of these identified antigens belong to one of the following categories according to expression patterns or structural features: a) cancer-testis (CT) antigens, such as the members of the *MAGE* gene family, SSX2, SCP1, and NY-ESO-1 (Old LJ, 2001); b) mutated antigens, eg, p53 (Theobald et al, 1995) and CDK4 (Rosenberg, 1997); c) overexpressed antigens, eg, HER-2/neu (Offringa et al, 2000) and PRAME (Ikeda et al, 1997); d) differentiation antigens, such as Melan-A/MART1, tyrosinase, and gp100 in melanocytes (Palermo et al, 2001); and e) virus antigens, such as hepatitis B and C viruses, in hepatocellular carcinoma (HCC) (Zondervan et al, 2000), and human papilloma virus in cervical cancer (Sellors et al, 2003). Because of their characteristic expression patterns in cancer and testis, not in other normal tissues, CT antigens are of major interest as targets for immunotherapy and may also serve as tools for diagnostic purposes.

HCC is one of the most prevalent malignancies, with an increasing incidence worldwide, especially in East Asian countries (Okuda, 2000). In view of the poor prognosis for HCC patients with surgery and chemotherapies, an alternative of immuno- and gene-therapeutic strategies has been pursued in patients to improve the treatment (Hanke et al, 2002; Reinisch et al, 2002). A prerequisite for the success of bio-therapeutic strategies is the identification of the genes or antigens that are exclusively or preferentially expressed in malignant tissues. With the application of serological analysis of recombinant cDNA libraries (SEREX), several tumor-associated antigens have been identified in HCC by other groups (Stenner-Liewen et al, 2000; Wang et al, 2002) and our group. We have identified two novel CT antigens in HCC, one of which is HCA587. Investigating the immunogenicity of HCA587 will help us to understand if it is a potential target for immunotherapy and diagnosis in cancer patients.

By RT-PCR, we have demonstrated that HCA587 mRNA is highly expressed in HCC (Wang et al, 2002). However, we don't know the extent of HCA587 protein expression in HCC cells, and it is an essential condi-

DOI: 10.1097/01.LAB.0000080605.73839.96

Received April 20, 2003.
This work was supported by grants from National 973 Program in China (No. G1999053904), Ludwig institute for Cancer Research (KSP003), National 863 program in China (No. 2001AA215411), and Beijing Municipal Government Foundation for Natural Sciences (No. 7001002).
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Possible explanations are: (1) the host phylogeny is in error; (2) the *Kryptophanaron* symbiont became obligate and was isolated from free-living populations earlier than the *Anomalops* and *Photoblepharon* symbionts; or (3) the symbiont of either *Anomalops* or *Photoblepharon* displaced the other when the two host genera became sympatric, and subsequently diverged along with their hosts. A molecular phylogeny of the host family may help to clarify the issue. Another surprising observation is that symbionts of two specimens of *P. palpebratus* are quite distinct (Fig. 3). In contrast, six *K. alfredi* from two locations in the Caribbean and *A. katoptron* from two locations were identical within their respective host genera[15]. The *P. palpebratus* samples came from two morphological types from the same location, and may reflect an unrecognized taxonomic difference in the hosts.

Received 1 December 1992; accepted 22 March 1993.

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ACKNOWLEDGEMENTS. We thank D. Lane for *Vibrio* sequences, R. Rosson for *V. cholerae* sequence and discussions, R. Rosson and K. Nealson for luminous bacterial sequences, P. Colin, A. C. Ameson and J. McCosker for fish samples, D. Edwards for technical assistance, D. Davison and G. Olsen for help with fastDNAml and E. DeLong for discussions and unpublished sequence data. This work was supported by a University of California Faculty Career Development Award to M. G. H. and US Office of Naval Research grants to M.G.H. and R. Rosson and K. Nealson.

Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells

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ACTIVATION of CD4$^+$ T helper cells results from the occupancy of the T-cell receptor (TCR) by immunogenic peptide bound to a class II major histocompatibility complex (MHC) molecule[1], together with a co-stimulatory signal from the antigen-presenting cell (APC)[2]. This activation leads to proliferation, cytokine production (Th1 or Th2 profile) and cytolysis[3]. Engagement of the TCR in the absence of co-stimulation causes Th1 cells to become unresponsive to subsequent antigenic stimulation[4–6]. We have previously demonstrated that analogues of an immunogenic peptide could stimulate Th1 and Th2 cells to carry out some effector functions without inducing proliferation[7,25], a phenomenon we term partial activation. Here we study the consequences of such partial activation through the TCR of two Th1 clones using peptide analogues presented by a live APC. A peptide analogue that is unable to stimulate clonal proliferation or production of cytokine or inositol phosphate can induce the T cells to become profoundly unresponsive to subsequent stimulation with the immunogenic peptide. Thus, altering the ligand of the TCR by using a peptide analogue on a functional APC sends a signal to Th1 clones that results in anergy.

The Th1 clone PL.17 is specific for the immunogenic peptide of the murine haemoglobin β^d minor chain, Hbβ(64–76), bound to an I-E^k molecule. Peptide analogues were constructed by introducing conservative, single amino-acid substitutions at the previously identified TCR contact positions of this peptide[8]. Using an I-E^k-transfected fibroblast line, DCEK Hi7 (ref. 9), as APC, we analysed the functional responses of PL.17 cells after stimulation with the immunogenic peptide or analogues containing substitutions of serine for alanine at position 70 (Ser 70), or of glutamine for asparagine at position 72 (Gln 72). These analogues bind to I-E^k molecules with an affinity comparable to the Hb(64–76) peptide, as evidenced by their effective competition with ^{125}I-labelled Hb(64–76) in a direct binding assay and with another I-E^k-restricted peptide in a functional assay[8]. The Ser-70 and Gln-72 analogues did not induce T-cell proliferation, or production of Th1 cytokines (interleukins IL-2 and IL-3, and interferon-γ) or free inositol phosphates (Fig. 1; IL-3 data not shown). Parallel experiments using B10.BR (Hbβ^s) spleen cells as APC gave similar results.

Upon activation, T cells upregulate various cell-surface molecules, including the IL-2 receptor and lymphocyte function antigen LFA-1 (refs 10, 11). Flow cytometric analysis revealed that the Ser-70 analogue, like the immunogenic peptide, stimulated upregulation of both the IL-2 receptor (Fig. 2*a*) and LFA-1 (Fig. 2*b*). Surface expression of other molecules, including CD3 and class I major histocompatibility complex (MHC), did not change. Stimulation with the Gln-72 analogue did not generally lead to upregulation of the IL-2 receptor or LFA-1, although each surface molecule was increased slightly in two of six experiments. Consistent with T-cell stimulation[5], PL.17 cells activated with the Ser-70 analogue, but not the Gln-72 analogue, showed a significant cell volume increase by flow cytometric analysis (data not shown). Thus, the Ser-70 analogue was delivering a signal to PL.17 upon TCR engagement which allowed upregulation of IL-2 receptors and of LFA-1, and increased cell volume, but did not activate cytokine production or proliferation.

These interesting properties of the Th1 clone PL.17 and the Ser-70 analogue peptide enabled us to evaluate whether TCR engagement without proliferation leads to anergy[12]. We used a proliferation assay to study PL.17 previously stimulated with DCEK Hi7 or B10.BR spleen cells as a source of live APC, either alone or with peptide analogues. PL. 17 cells cultured with APC alone, or APC and the Gln-72 analogue, proliferated vigorously upon subsequent stimulation with Hb(64–76). But PL.17 cells first cultured with APC and the Ser-70 analogue were now completely unresponsive to the immunogenic peptide (Fig. 3*a*, *b*). These PL.17 cells were tolerant to stimulation with Hb(64–76), but the proliferation response was normal to exogenous IL-2 (Fig. 3 legend) or to phorbol myristate acetate and Ca^{2+} ionophore (data not shown). When B10.BR lipopolysaccharide-treated spleen cell blasts and B-cell lymphomas were used as sources of live APC, we still found Ser-70 analogue-induced anergy of PL.17. There were two other important results. First, preincubation of another Hb(64–76)-specific Th1 clone, Hb#2, with the Ser-70 analogue also resulted in unresponsiveness

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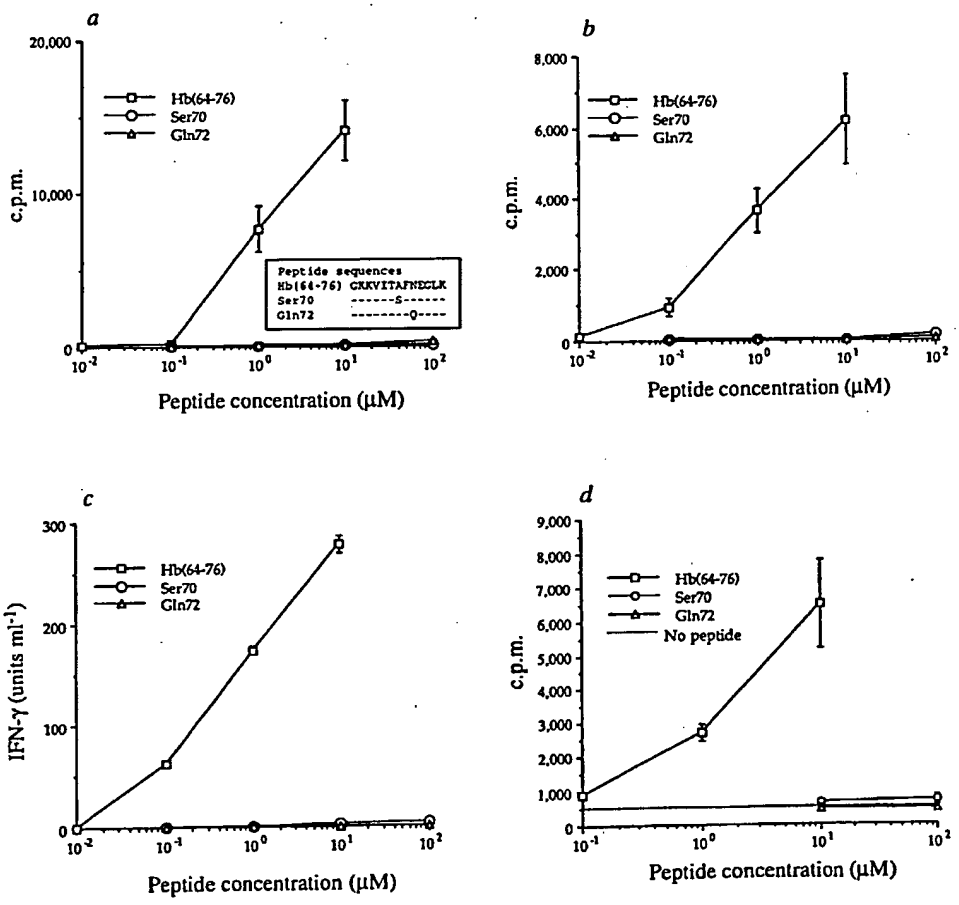
to the immunogenic peptide (Fig. 3c). However, the Ser-70 analogue stimulated a cytolytic response, but not a proliferative response, from Hb#2 cells[25], an indication that this peptide analogue caused partial activation of this T-cell clone. Second, the Ser-70 analogue is capable of causing proliferation of other haemoglobin-specific T-cell clones (2 of 6 clones)[8]. This indicates that the interaction between TCR and the peptide–MHC complex, and not the peptide itself, is what determines the subsequent T-cell response. We are now investigating T-cell receptor usage among these different clones to see whether there is any correlation with the anergic/stimulatory response.

We showed that this tolerance of T cells was not due to carryover of APC presenting Ser-70 analogue or to temporary refractoriness of the T cells in the following experiments. To eliminate the effect of analogue carryover, graded doses of APC preincubated with the Ser-70 analog (100 μM) were added simultaneously with fresh APC and Hb(64–76), and there was no inhibition of proliferative response of PL.17 (<5% of the response). To see whether there was temporary refractoriness of T cells after antigen stimulation[13,14], we incubated PL-17 cells with either a proliferative dose of Hb(64–76) (10 μM) or a tolerogenic dose of Ser-70 analogue (50 μM) and compared their responses to immunogenic peptide after 7 days. As the T cells could mount a proliferative response after incubation with Hb(64–76) (58,974 c.p.m.) but not after incubation with the Ser-70 analogue (10,039 c.p.m.), they were not refractory to antigen restimulation at this time point.

Previous studies using chemically fixed spleen cells and immunogenic peptide indicated that the unresponsive state of T cells lasted for a relatively long time (tested up to 8 days)[4]. Addition of cyclosporin A (CsA) or an exogenous source of costimulation to the culture prevented the T cells from becoming anergic[15,16]. Similarly, the Ser-70 analogue also induced long-lasting anergy in PL.17 because the T cells were still unresponsive



1 Proliferation, lymphokine production and inositol phosphate generation responses of PL.17 clone to Hb(64–76) and its analogue peptides. *a*, Proliferation response; *b*, production of IL-2 and, *c*, interferon (IFN)-γ; and *d*, inositol phosphate generation induced by Hb(64–76), and its Ser-70 and Gln-72 peptides.

METHODS. Proliferation was assayed in 96-well flat-bottomed plates in 200 μl RPMI-1640 medium containing 10% fetal calf serum (Hyclone), 2 mM glutamine, 50 μg ml^{-1} gentamycin, 10 mM HEPES and 2-mercaptoethanol (2×10^{-5} M). The following were added to the appropriate wells: PL.17 clone, at 2×10^{4} cells per well, mitomycin C-treated DCEK Hi7 fibroblasts (77 μg ml^{-1}) in Hank's balanced salt solution for 90 min at 37 °C (Sigma) transfected with I-E^{k} construct[9] or CH27 B lymphoma cells (for IL-2 assay) at 5×10^{4} cells per well, and Hb peptides (0–100 μM). The assay was incubated at 37 °C for 72 h with addition of ^{3}H-thymidine (0.4 μCi per well) during the last 20 h. Lymphokine responses were assessed using 24-h (IL-2) or 48 h (IFN-γ) supernatants from these cultures. IL-2 was quantitated in a bioassay as proliferation of the IL-2-dependent cell line CTLL-2 (ref. 21) as described[22]. IFN-γ was measured by ELISA using reagents provided by R. Schreiber[23]. Monoclonal antibody H22 was used to capture IFN-γ from test supernatants, and bound cytokine was identified with a polyvalent rabbit anti-murine IFN-γ, followed by peroxidase-conjugated goat anti-rabbit IgG (TAGO). The substrate was developed with ABTS (2,2'-azino-di-[3-ethyl-benzthiazolinsulphonate(b)]) reagent and the absorbance read at 414 nm. For inositol phosphate detection, PL.17 cells were incubated overnight at $1–2\times10^{7}$ cells per ml in inositol-free RPMI complete medium containing 20–50 μCi ml^{-1} *myo*-[2-^{3}H]inositol (Amersham). Cells were then washed in HBSS and resuspended in medium with 10 mM LiCl (inositol phosphatase inhibitor). T cells were incubated in 96-well flat-bottomed plates ($7–10\times10^{5}$/well) with the indicated doses of Hb(64–76) or peptide analogue and DCEK Hi7 ($7–10\times10^{5}$ per well) for 90–120 min and assayed for accumulation of free inositol phosphates as described[24]. Briefly, cultures were extracted with 1 ml of a 1/2 mixture of chloroform and methanol followed by 0.25 ml chloroform and H_2O. Phases were separated by centrifugation and the H_2O-soluble fraction run on a 0.25 ml AG1-X8 formate ion-exchange column (BioRad), then washed extensively with 5 mM *myo*-inositol. Total free inositol phosphate was eluted from the column with 0.1 M formic acid, 1 M sodium formate in 1.5 ml, and the radiolabel quantitated by scintillation counting. IL-3 was quantitated using a bioassay[25]. For some proliferation and lymphokine assays, DCEK Hi7 cells were substituted by irradiated B10.BR/SgSnJ spleen cells (2,000 rads, 5×10^{5} cells per well). The Hbβ(64–76) peptide and peptide analogue were synthesized using a DuPont RaMPS apparatus, purified by reverse-phase HPLC, analysed and quantitated using a Beckman 6300 amino-acid analyser. The sequence of the Hb(64–76) peptide and the analogues substitutions are shown in the insert to *a*. Experiments were done in triplicate, or in duplicate for inositol phosphate generation; error bars represent standard deviation of the mean. Each experiment is representative of 4–5 independent assays.

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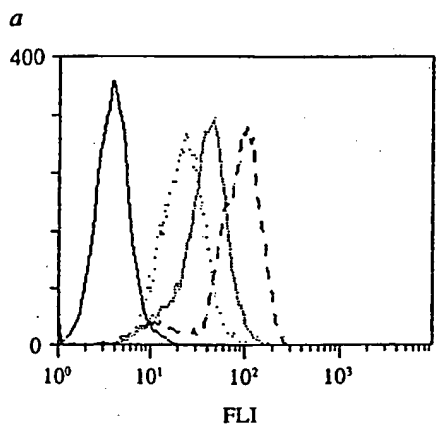
to the immunogenic peptide seven days after culture with this analogue (Fig. 4*a*). CsA addition with the Ser-70 analogue prevented induction of anergy (Fig. 4*b*). But, in contrast to the anergy induced on chemically fixed APC, in this system addition of allogeneic spleen cells depleted of T lymphocytes as a source of co-stimulation had no effect (data not shown). Furthermore, anergy of PL.17 was not induced by presentation of the Ser-70 analogue on chemically fixed APC. These observations suggest that anergy induced by stimulating peptides with fixed APC is different from that induced by analogues with live APC.

Our finding that T cell anergy can occur by presentation of peptide analogues on a live functional APC is not the same



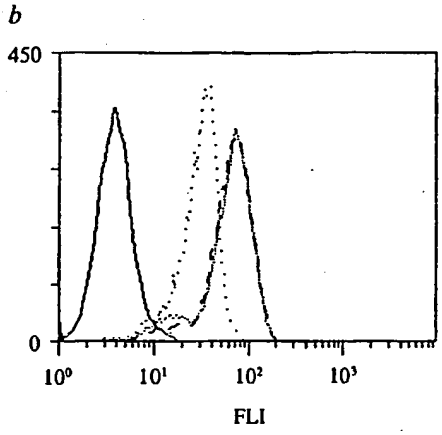
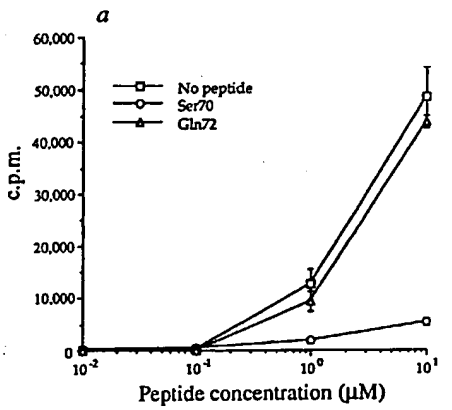
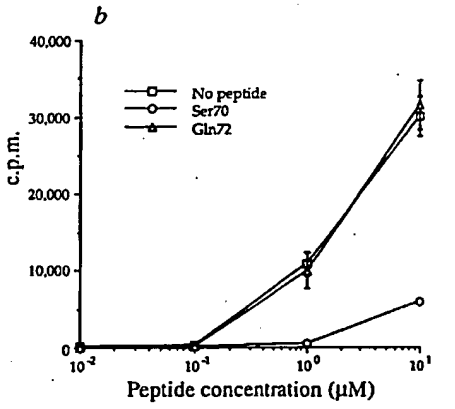


FIG. 2 FACS analysis reveals that the Ser-70 analogue induces upregulation of *a*, the IL-2 receptor and *b*, LFA-1 on PL.17; *x*-axis represents fluorescence intensity (FLI) and *Y*-axis represents relative cell number.
METHODS. PL.17 (5×10^5 cells per well) were incubated with DCEK Hi7 L cells (5×10^5 per well) alone (· · ·) or with 50 μM Ser70 analog (· · ·) or 0.01 μM Hb(64–76) (– – –) in 24-well plates for 48 h. T cells were then separated from L cells by centrifugation over Ficoll-Hypaque (Pharmacia) at 3,000 r.p.m. for 15 min, and washed 3 times before FACS analysis. For each group, 1×10^5 cells were incubated with anti-IL-2 receptor (*a*; 7D4, rat IgG) or anti-LFA-1 (*b*; FD441.8, rat IgG), and captured using a fluorescein isothiocyanate-labelled goat anti-rat IgG (Southern Biotech). PL.17 cells, preincubated with DCEK Hi7 cells alone, were stained with goat anti-rat IgG alone as a negative control (——). Cells were analysed on a FACScan (Becton Dickinson). Data are representative of 4 different experiments.





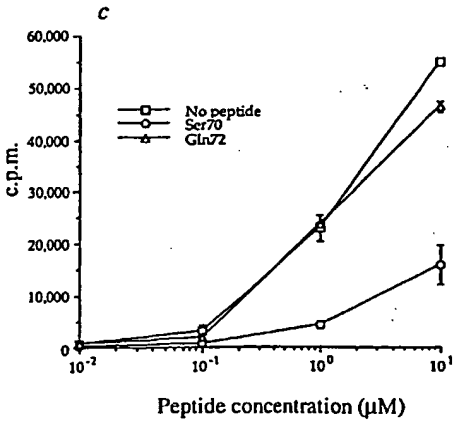


FIG. 3 Ser-70 analogue induces anergy of PL.17 and Hb2. Th1 cells (5×10^5 per well) and mitomycin C-treated APC were incubated alone or with 50 μM Ser-70 analogue or 50–100 μM Gln-72 analogue for 20–24 h at 37 °C in 24-well tissue culture plates in a final volume of 700 μl. T cells were then separated from APC as for Fig. 2, and rested for 2–3 days in 48-well tissue culture plates in RPMI medium before challenge in a proliferation assay (Fig. 1 legend) using Hb(64–76) as antigen, or with IL-2 (20 units per well). T cells and APC were used as follows: *a*, PL.17 Th1 cells and DCEK Hi7 L cells (5×10^5 per well); *b*, PL.17 Th1 cells and B10.BR/SgSnJ spleen cells (5×10^6 per well); *c*, Hb2 Th1 cells and DCEK Hi7 L cells. Cell recovery after the rest period, expressed as a percentage of input cell number, was: *a*, 50% for all three groups; *b*, 60% for no-peptide and Gln-72 groups, and 50% for Ser-70 group; *c*, 75% for no-peptide group, 90% for Ser-70 group, and 83% for Gln-72 group. IL-2 responses were: *a*, no peptide, 12,270 ± 2,493 c.p.m.; Ser-70 Hb(64–76), 15,751 ± 689 c.p.m., and Gln-72 Hb(64–76), 10,864 ± 843 c.p.m. *b*, No peptide, 18,061 ± 6,468 c.p.m.; Ser-70 Hb(64–76), 29,447 ± 5,738 c.p.m., and Gln-72 Hb(64–76), 11,816 ± 2,365 c.p.m. *c*, No peptide, 70,543 ± 4,009 c.p.m.; Ser-70 Hb(64–76), 66,654 ± 6,766 c.p.m., and Gln-72 Hb(64–76), 50,929 ± 3,857 c.p.m. In some cases, DCEK Hi7 L cells or CH27 cells (5×10^4) replaced the spleen in the challenge assay, giving similar results. Data points represent the mean of triplicate cultures ±s.d. Each experiment is a representative from 3 (*b*), 4 (*c*) or 9 (*a*) independent assays.

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phenomenon as a previously described peptide antagonism that has been explained in terms of a stochastic model of competition for TCR sites by analogue peptides. In this system[17], inhibitory antigen analogues cannot induce T cell tolerance; in our system, competition for TCR sites is not implicated in the lack of response because the analogue peptide is removed before stimulation with immunogenic peptide. A peptide analogue may interact with lower affinity with the TCR, so only part of the normal signal is delivered upon TCR engagement. This could result because of rapid dissociation of the TCR from the peptide-MHC complex, or a lack of allosteric changes in the TCR, or because there is no association of surface regulatory molecules with the TCR, or combinations of these factors. This partial signal, in the absence of a full stimulatory signal, results in T-cell anergy rather than clonal expansion. As the TCR/CD3 complex has multiple chains that are thought to be involved in signal transduction upon ligand interaction, some, but not all, of these chains may be engaged upon presentation of anergy-inducing peptide analogues. Indeed, some chains of the CD3 complex can transduce signals independently of the others[18-20]. These results were not peculiar to this particular peptide analogue, because preincubation with the Asp-73 analogue, which also fails to stimulate PL.17 proliferation, inhibits the proliferative response to Hb(64-76) (77% inhibition). We have observed a similar pattern of reactivity for several T-cell clones.

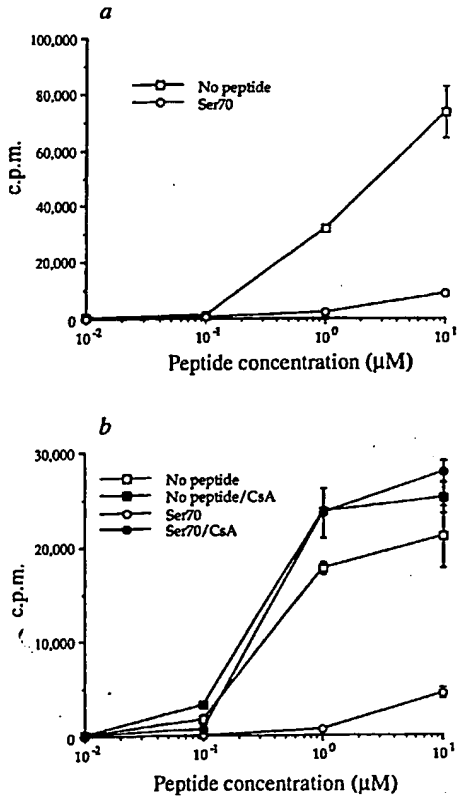

FIG. 4 Ser-70 analogue-induced anergy is *a*, long-term and *b*, prevented by cyclosporin A.
METHODS. PL.17 cells were used in the tolerance assay with DCEK Hi7 L cells as described for Fig. 3a, with the addition of cyclosporin A (1 μg ml^{-1}) in *b*. T cells were separated from APC as described in Fig. 2 legend and were then rested in 48-well tissue culture plates for *a*, 7 days, or *b*, 3 days, before being used in the challenge assay described in Fig. 3 legend. Data points represent means of triplicate cultures ±s.d. Each experiment is representative of 5 (*a*) or 3 (*b*) independent assays.

(J.S.L., unpublished observations). Our results show that, by introducing conservative amino-acid substitutions in the TCR contact residues of an immunogenic peptide, the interaction of the ligand can be changed so that only part of the signal is delivered. The extent of the signal transduced appears to be dependent on which TCR contact residue is altered, with changes in more permissive residues inducing T-cell anergy, and changes in less permissive residues preventing the delivery of any signal to the T cell. Most ligands for T cells should be amenable to such manipulation, an approach that may eventually have clinical application.

Note added in proof: In another system, evidence for ligand-related differences in T-cell receptor-dependent intracellular signalling was recently demonstrated by peptide-MHC class II complexes with mixed agonist/antagonist properties[26]. □

Received 21 January; accepted 2 March 1993.

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ACKNOWLEDGEMENTS. We thank E. R. Unanue, O. Kanagawa and J. Braun for critical reading of the manuscript. This work was supported by grants from the NIH.

Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide

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SEVERAL observations suggest interactions between the immune and nervous systems[1,2]. Psoriasis and atopic dermatitis may worsen with anxiety and have been associated with anomalous neuropeptide regulation[2]. Neurotransmitters affect lymphocyte function[1-4] and lymphoid organs are innervated[5-9]. Calcitonin gene-related peptide (CGRP) is a neuropeptide and vasodilator[10] that modulates some macrophage functions, including antigen presentation *in vitro*[11]. CGRP is associated with Langerhans cells (LC) in oesophageal mucosa, particularly during inflammation[12], is present in epidermal nerves and is associated with Merkel cells[10,13-15]. We

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